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13. ABSTRACT (Maximum 200 Words) The purpose of this project is to generate monoclonal antibodies to receptor molecules for the angiogenic growth factor VEGF. These antibodies will be tested for their potential as inhibitors of VEGF-stimulated angiogenesis, which is thought to play an important role in the development of breast cancer. A number of hybridoma fusions have been done, but screening has been delayed. Research defining the VEGF binding site of the Flt-1 receptor has been published; this work will aid in the selection of receptor fragments for immunization. Research elucidating two signaling pathways required for VEGF-stimulated endothelial cell proliferation has been published; a conclusion of that study is that therapeutic intervention of VEGF-induced angiogenesis should focus on early signaling events.					
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Introduction

The survival and growth of solid tumors is dependent on the neo-vascularization of the growing tumor mass. Prior to the vascularization of a tumor, a subpopulation of tumor cells acquire an angiogenic phenotype characterized by the production of polypeptide growth factors that stimulate endothelial cell proliferation, migration and differentiation. By activating cell surface receptor molecules, these tumor-derived angiogenic factors induce the growth of blood vessels into the tumor from pre-existing blood vessels. Thus, the process of tumor angiogenesis represents an attractive target for the development of new cancer therapeutic agents. We are characterizing the signaling pathways utilized by vascular endothelial growth factor (VEGF), an angiogenic factor that is widely expressed by breast tumors, and we are generating neutralizing monoclonal antibodies to receptor molecules for VEGF. We will test the antibodies for the ability to block VEGF-induced endothelial cell proliferation and capillary growth in vitro.

Body of Report

Progress in the second year of this grant has been impacted by the relocation of the Principal Investigator in 1999 from the Adirondack Biomedical Research Institute to the American Type Culture Collection (ATCC), a not for profit bioscience institution in Manassas, VA, where the P.I. occupies a laboratory on the Prince William Campus of George Mason University. The grant was transferred to the ATCC in July 2000. Despite this hiatus, the following work was accomplished.

Specific Aim 1: Tasks 1-3

Vascular endothelial cell growth factor (VEGF) stimulates human endothelial cell proliferation by binding to two cell surface receptor molecules: Flt-1 (*fms*-like tyrosine kinase) and KDR (kinase insert domain-containing receptor) (1,2). Davis-Smyth, et al. (3) have used domain swapping between Flt-1 and a related receptor molecule, Flt-4, that does not bind VEGF to localize the major VEGF binding region of Flt-1 to the second of seven extracellular immunoglobulin-like loops. As part of this project, we have examined cell-bound receptor chimeras consisting of fragments of the extracellular region of Flt-1 and the non-related cell surface molecule embigin to further characterize the VEGF-binding region of Flt-1 (4, Appendix 1). Our results indicate that either immunoglobulin-like loop 1 or 3 of Flt-1 is required in addition to loop 2 for high affinity VEGF binding. These findings in conjunction with the work of others (5,6) localize the VEGF binding region of Flt-1 to extracellular immunoglobulin-like loops 1 to 3. We will incorporate these results into our receptor immunization protocol by immunizing mice with insect cells expressing recombinant Flt-1 constructs consisting of Flt-1 loops 1-3 fused to embigin (4). High 5 cells infected with baculovirus bearing chimeric Flt-1/embigin cDNA have been stockpiled for this purpose. The animals will be boosted with purified Flt-1/embigin chimeric molecules.

We have hybridomas from four fusion experiments with spleen cells immunized with full length recombinant human Flt-1. These fusion products will be screened for neutralizing antibody activity by competitive binding with radioactive VEGF once an institutional license for the use of radioactive materials is granted to George Mason University by the Nuclear Regulatory Commission. In anticipation of screening, we have constructed, expressed and purified mutant forms of VEGF that selectively bind to either Flt-1 or KDR; the mutations used were based on an analysis of receptor binding regions of VEGF by Keyt, et al. (7). These mutant VEGFs will allow us to screen for antibodies to a single receptor type using human endothelial cells that express both receptor types.

Specific Aim 2: Task 7

We have done a pilot experiment aimed at developing an in vitro microvessel growth assay. A piece of human umbilical cord was cut into small fragments which were plated on a collagen-coated substratum in several different media. Initially DME/F12 nutrient medium was supplemented with either 10% fetal bovine serum, 10 ng/ml fibroblast growth factor-2 (FGF-2), 10 ng/ml VEGF or FGF-2 and VEGF. After a growth phase in which cells migrated out of the tissue fragments and proliferated on the substratum, the nutrient medium supplemented with the purified growth factors was changed to MCDB 153, which possesses a low calcium concentration. A large percentage of cells died in the new medium, which was consistent with the inability of fibroblast cells to grow under conditions of low calcium (8). The remaining cells had the morphological characteristics of endothelial cells, but they have not yet been tested for biochemical markers of endothelial cells. These cells were not embedded in a collagen gel and therefore did not form tube structures. Further experiments will be done with umbilical tissue obtained from the Prince William Hospital in Manassas, VA; a revised human subjects protocol will be reviewed by the PWH Human Subjects IRB on Aug. 25.

The strategy of inhibiting the angiogenic activity of VEGF at the level of ligand-receptor interactions at the cell surface rather than at the level of intracellular signaling pathways is supported by our study of intracellular pathways that are activated in endothelial cells during a mitogenic response to VEGF (9, Appendix 2). We found that the mitogen-activated protein (MAP) kinases ERKs 1 and 2, and p38 MAP kinase but not Jun N-terminal kinase (JNK) were activated in response to VEGF. In addition phosphatidylinositol 3'-kinase (PI 3-kinase) signaling through p70 S6 kinase was activated. By using specific inhibitors of these kinases, we showed that activation of ERKs 1 and 2 and the PI 3-kinase/p70 S6 kinase pathway were both required for endothelial cells to proliferate in response to VEGF. However, these pathways were not uniquely activated by VEGF but were also activated by receptors for epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2). This observation of convergent growth factor signaling in endothelial cells indicates that therapeutic strategies aimed at inhibiting intracellular signaling components will not be specific for responses to VEGF. This conclusion is a compelling argument for focusing on proximal steps in VEGF signaling to inhibit angiogenesis.

We have extended our study on VEGF signaling in endothelial cells to show that PI 3-kinase, a signaling enzyme whose activity is required for a mitogenic response to VEGF, binds directly to the Flt-1 receptor for VEGF (10). This direct interaction is significant in that it implies that PI 3-kinase is activated through VEGF binding to Flt-1. To date PI 3-kinase activation has only been experimentally linked to the KDR receptor (11). Thus, it is likely that dual receptors signals are involved in PI 3-kinase activation.

Key Research Accomplishments

- Characterization of the minimal high affinity VEGF binding site on the Flt-1 receptor
- Identification of required signaling pathways in VEGF-induced cell proliferation
- Direct demonstration of PI 3-kinase binding to Flt-1 VEGF receptor

Reportable Outcomes

Publications

1. Herley, M.T., Yu, Y., Whitney, R.G., and Sato, J.D. (1999) Characterization of the VEGF binding site on the Flt-1 receptor. *Biochem. Biophys. Res. Commun.* 262: 731-738.
2. Yu, Y., and Sato, J.D. (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. *J. Cell. Physiol.* 178: 235-246.
3. Yu, Y., and Sato, J.D. (2000) VEGF signaling pathways in endothelial cell mitogenesis. DoD Breast Cancer Research Program Era of Hope Meeting. Atlanta, GA, June 8-12, 2000, p. 568.

Conclusions

The major conclusion of the research done thus far is that because signaling pathways initiated by different growth factors converge or overlap, therapeutic interventions of angiogenesis or other growth factor-induced processes should target early events in signaling cascades in order to achieve the greatest degree of specificity.

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Appendices

Appendix 1

Characterization of the VEGF binding site on the Flt-1 receptor.
Biochem. Biophys. Res. Commun. 262: 731-738 (1999).

Appendix 2

MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. 178: 235-246 (1999).

Appendix 3

VEGF signaling pathways in endothelial cell mitogenesis. DoD Breast Cancer Research Program Era of Hope Meeting. Atlanta, GA, June 8-12, 2000, p. 568.

Characterization of the VEGF Binding Site on the Flt-1 Receptor

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The angiogenic growth factor VEGF binds to the receptor tyrosine kinases Flt-1 and KDR/Flk-1. Immunoglobulin (Ig)-like loop-2 of Flt-1 is involved in binding VEGF, but the contribution of other Flt-1 Ig-loops to VEGF binding remains unclear. We tested the ability of membrane-bound chimeras between the extracellular domain of Flt-1 and the cell adhesion molecule embigin to bind VEGF. VEGF bound as well to receptors containing Flt-1 loops 1–2 or 2–3 as it did to the entire Flt-1 extracellular domain. Chimeras containing only loop-2 of Flt-1 bound VEGF with 22-fold lower affinity. We conclude that high-affinity VEGF binding requires Ig-like loop-2 plus either loop-1 or loop-3. In addition, Flt-1 amino acid residues Arg-224 and Asp-231 were not essential for high-affinity binding of VEGF to membrane-bound Flt-1. © 1999 Academic Press

Vascular endothelial growth factor (VEGF) is an angiogenic heparin-binding growth factor (Senger *et al.*, 1983; Ferrara and Henzel, 1989). VEGF functions through binding to its receptor tyrosine kinases Flt-1 (Shibuya *et al.*, 1990; DeVries *et al.*, 1992) and KDR/Flk-1 (Matthews *et al.*, 1991; Terman *et al.*, 1992), thus activating intracellular signaling cascades (Myoken *et al.*, 1991; Waltenberger *et al.*, 1994; Cunningham *et al.*, 1997a; Yu and Sato, 1999). Whereas other angiogenic factors are pleiotrophic, VEGF is mitogenic specifically for endothelial cells. This selectivity is achieved in part by the restricted expression of the VEGF receptors: KDR expression is limited to endothelial cells, whereas Flt-1 is expressed by both endothelial cells and monocytes (Quinn *et al.*, 1993). Thus, human monocytes express Flt-1, but not KDR (Barleon *et al.*, 1996; Clauss *et al.*, 1996). VEGF induces monocyte migration, but not proliferation, indicating that Flt-1 and KDR may have distinctly different roles *in vivo*.

Angiogenesis plays a key role in many physiological and pathological conditions, including wound healing, embryonic development, tumor growth, diabetic retinopathy and rheumatoid arthritis (Folkman, 1995; Risau, 1997). Anti-VEGF monoclonal antibodies which inhibit VEGF/VEGF receptor interaction have been used to inhibit tumor growth in mice (Kim *et al.*, 1993) and have since been humanized for use in clinical trials (Presta *et al.*, 1997). The importance of VEGF, Flt-1, and KDR for developmental angiogenesis is emphasized by the results of the targeted disruption of these genes in mice. Mice lacking even a single VEGF allele die *in utero* at days 11–12 postcoitum (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996), indicating a strong dose-dependence for VEGF during mouse embryonic development. Mice lacking either Flt-1 or Flk-1 (the mouse homolog of KDR) also die *in utero*, between days 8.5–9.5. Mice lacking Flk-1 failed to produce hematopoietic and endothelial cell precursors (Shalaby *et al.*, 1995), whereas Flt-1^{−/−} mice formed differentiated endothelial cells but had disorganized embryonic vasculature (Fong *et al.*, 1995). This indicates that Flt-1 and KDR have distinct and unique functions, which are essential for normal vascular development.

There have been conflicting reports regarding the minimal region of Flt-1 that contains the VEGF binding site. Loop-2 of Flt-1 was sufficient for high affinity VEGF binding in the context of an Flt-1/Flt-4 membrane-bound chimeric receptor protein (Davis-Smyth *et al.*, 1996). However, soluble chimeric or truncated Flt-1 proteins required a minimum of loops 1–2 (Cunningham *et al.*, 1997b) to loops 1–3 (Davis-Smyth *et al.*, 1996; Barleon *et al.*, 1997) to support full VEGF binding. In order to identify the minimal region of membrane-bound Flt-1 required for VEGF binding without using sequence from the homologous receptor Flt-4, we made fusion proteins between Flt-1 and the unrelated Ig-loop-containing cell adhesion molecule embigin (Guenette *et al.*, 1997). By using embigin instead of the more closely related receptor Flt-4, we

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TABLE 1
DNA Sequence of Oligonucleotides Used in This Study

Primer	Oligonucleotide sequence	Restriction site
V-3A	5'-ACCATGACCTTTCTGCTGTCTTGG-3'	
V-1B	5'-TGGTGAGAGATCTGGTTCCCGAAA-3'	
F-A1A	5'-ATCCTCGAGCGCTCACCATGGTCAGCTAC-3'	<i>Xho</i> I
F-4B	5'-CCACTTGCTGGCATCATAAGG-3'	
1392-for	5'-CCGGATTATTCATACCGTC-3'	
1392-rev	5'-CGGATTTCCCTGAAGAGAG-3'	
f-link-e	5'-CTGCATATGGTC-TACCTGTTTCGAGAG-3'	<i>Nde</i> I
E800F	5'-CTAGTTAACGCAGCC-TTCCCGTTAGG-3'	<i>Hin</i> CII
E611F	5'-GACCTTAAGGATTCTACT-GTGCTGAAGTG-3'	<i>A</i> flII
F962F	5'-GTCCTTAAGTCGTGTGT-GCTTATTTGGAC-3'	<i>A</i> flII
F475R	5'-GAAGATCTCCTTCCTTCA-GTCATGTG-3'	<i>Bgl</i> II
E323F	5'-GAAGATCTCACCTGGAACG-AATG-3'	<i>Bgl</i> II
F155F	5'-GCTCTAGATGCTTGCATGATGTGCTG-3'	<i>Xba</i> I
F440F	5'-GCTCTAGACCTTTCGTAGAGATGTACAG-3'	<i>Xba</i> I
R224AF	5'-CTCACACATGCACAAACCAATACAATCATAGATGTC-3'	
R224AR	5'-GGACATCTATGATTGTATTGGTTTGTGCATGTGTGAG-3'	
D231AF	5'-CCAATACAATCATAGCTGTCCAAATAAGCACACC-3'	
D231AR	5'-GGTGTGCTTATTTGGACAGCTATGATTGTATTGG-3'	

Note. Restriction sites introduced into oligonucleotides for the purpose of subcloning are underlined.

eliminated the possibility of introducing functionally redundant regions of Flt-4 into receptor chimeras.

The crystal structure of VEGF₈₋₁₀₉ has been determined in a complex with a soluble Flt-1 loop-2 fragment (Flt-1_{D2}) (Weismann *et al.*, 1997). The interface between Flt-1_{D2} and VEGF₈₋₁₀₉ was primarily hydrophobic and flat; the only direct polar interaction detected was between Asp-63 of VEGF and Arg-224 of Flt-1_{D2}. Previously, Asp-63 was among a cluster of three negatively charged VEGF amino acid residues found to be required for VEGF binding to Flt-1 (Key *et al.*, 1996). However, the significance of the electrostatic interaction between Asp-63 of VEGF and Arg-224 of Flt-1 was thrown into question by the ability of VEGF to bind to a soluble R224A Flt-1 receptor mutant in which Arg-224 was replaced by alanine (Davis-Smyth *et al.*, 1998). In this study, we ascertained the effect of two individual Flt-1 amino acid substitutions on VEGF binding to membrane-bound Flt-1 containing the entire extracellular domain.

MATERIALS AND METHODS

Reagents. Restriction enzymes were obtained from Promega (Madison, WI) and New England Biolabs (Beverly, MA). *Taq* polymerase was obtained from Fisher (Springfield, NJ), and Vent Exo⁺ polymerase was obtained from New England Biolabs. Grace's medium, yeastolate, lactalbumin hydrolysate and gentamicin were all obtained from Life Technologies (Gaithersburg, MD). SF9 and High-Five insect cells, and the baculovirus transfer vectors pVL-1392 and pBlueBac4 were obtained from Invitrogen (Carlsbad, CA). Baculogold DNA was obtained from Pharmingen (San Diego, CA). Na¹²⁵I was obtained from Amersham (Arlington Heights, IL). Fetal bovine serum and the anti-Flt-1 N-terminal specific pAb were obtained from Upstate Biotechnology (Lake Placid, NY). Oligonucleotides were synthesized by the Molecular Biology Core Facility at the Adirondack

Biomedical Research Institute (Lake Placid, NY). The oligonucleotides used in this study are listed in Table 1. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit from Stratagene (La Jolla, CA).

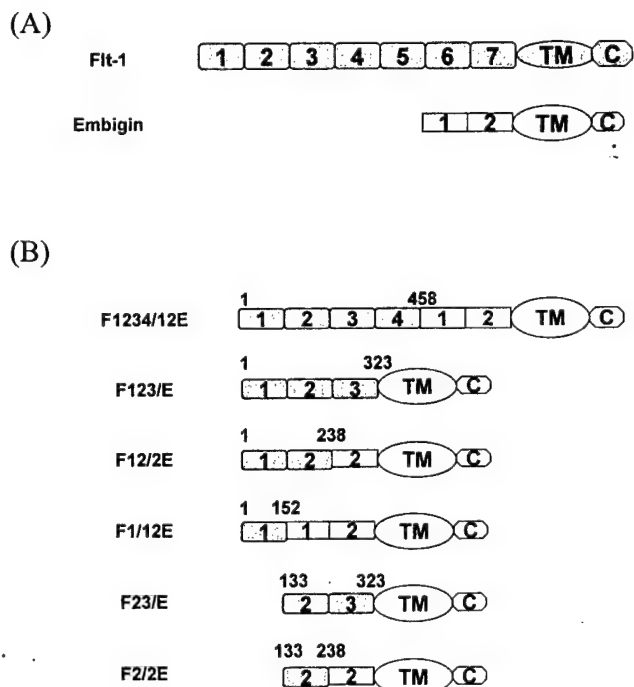


FIG. 1. Schematic representations of Flt-1, embigin, and Flt-1/embigin chimeras. (A) Human Flt-1 and rat embigin proteins. (B) Flt-1/embigin receptor chimeras. All receptors contained a single transmembrane domain (TM), and extracellular immunoglobulin (Ig)-like loops. All chimeric receptors contained embigin transmembrane and cytosolic domains (C).

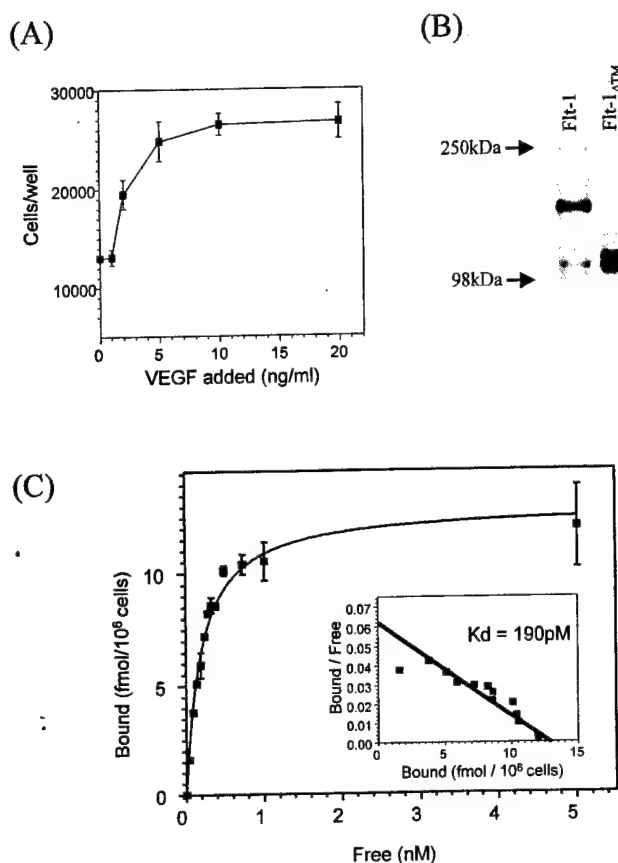


FIG. 2. Biological activities of recombinant VEGF and Flt-1 Δ TK. (A) HUVEC were incubated in the presence of increasing concentrations of recombinant VEGF. Cells were counted 5 days later. Error bars represent the range of duplicate determinations. (B) Flt-1, Flt-1 Δ TK and embigin were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with an anti-Flt-1 antibody. (C) Saturation binding of VEGF to High-5 cells expressing Flt-1 Δ TK. Error bars represent standard deviations ($n = 4$).

Cloning of VEGF, Flt-1, and embigin. VEGF cDNA was isolated from human A431 cells. cDNA amplified by PCR with the primers VEGF-3A and VEGF-1B was cloned into the pBlueScript-SK vector (Stratagene) and then transferred to the pVL-1392 baculovirus transfer vector (Invitrogen). Flt-1 cDNA was cloned from human umbilical vein endothelial cells (HUVEC) by PCR amplification. Flt-1 cDNA amplified with the PCR primers F-A1A and F-4B (Table 1) was cloned into pBlueScript-SK, cut with *Eco*RI and *Bam*HI and transferred to the pVL-1392 vector; this construct encoded a membrane-bound form of Flt-1 lacking the intracellular tyrosine kinase domain (Flt-1 Δ TK, nucleotides 1-2461). The isolation of rat embigin cDNA was described previously (Guenette *et al.*, 1997). Embigin cDNA was sub-cloned into the pVL-1392 transfer vector.

Flt-1/embigin chimera construction and expression. All chimeric receptor contained an Flt-1 signal sequence, and embigin transmembrane and cytosolic domains (Fig. 1). Fragments of Flt-1 cDNA generated either by PCR amplification with a 3:1 mixture of Vent(exo⁺) and Taq polymerases, or by restriction digests, were ligated to embigin cDNA as follows. **F1234/12E:** Flt-1 cDNA was cut with *Bgl*II and *Nde*I. Embigin cDNA was amplified by PCR with the oligonucleotides f-link-e and 1392-rev, cut with *Nde*I and *Eco*RI, then ligated to the Flt-1 *Bgl*II/*Nde*I cDNA fragment and to *Bgl*II/*Eco*RI cut pBlueBac4 vector. **F123/E:** Flt-1 cDNA was cut with *Not*I and *Hin*CII. Embigin cDNA was amplified with the PCR primers E800F

and 1392-rev, cut with *Hin*CII and *Eco*RI, and then ligated to the Flt-1 *Not*I/*Hin*CII cDNA fragment and to *Not*I/*Eco*RI cut pVL-1392 vector. **F12/2E:** Flt-1 cDNA was amplified by PCR with the primers 1392-for and F962R, and cut with *Not*I and *Afl*II. Embigin cDNA was amplified with the oligonucleotides E611F and 1392-rev, cut with *Afl*II and *Eco*RI, and then ligated to the Flt-1 *Not*I/*Afl*II cDNA fragment and to *Not*I/*Eco*RI cut pVL-1392 vector. **F1/12E:** Flt-1 cDNA was amplified by PCR with the oligonucleotides 1392-for and F475R, and then cut with *Not*I and *Bgl*II. Embigin cDNA was amplified with E323F and 1392-rev, cut with *Bgl*II and *Eco*RI, and ligated to the Flt-1 *Not*I/*Bgl*II cDNA fragment and to *Not*I/*Eco*RI cut PVL-1392 vector. **F23/E:** F123/E chimera cDNA was amplified by PCR with the oligonucleotides 1392-for and F155R, and the product cut with *Not*I and *Xba*I. F123/E chimera cDNA was also PCR-amplified with the oligonucleotides F440F and 1392-rev, and the product cut with *Xba*I and *Eco*RI. These two cDNA fragments were then ligated together and to *Not*I/*Eco*RI cut PVL-1392 vector. This

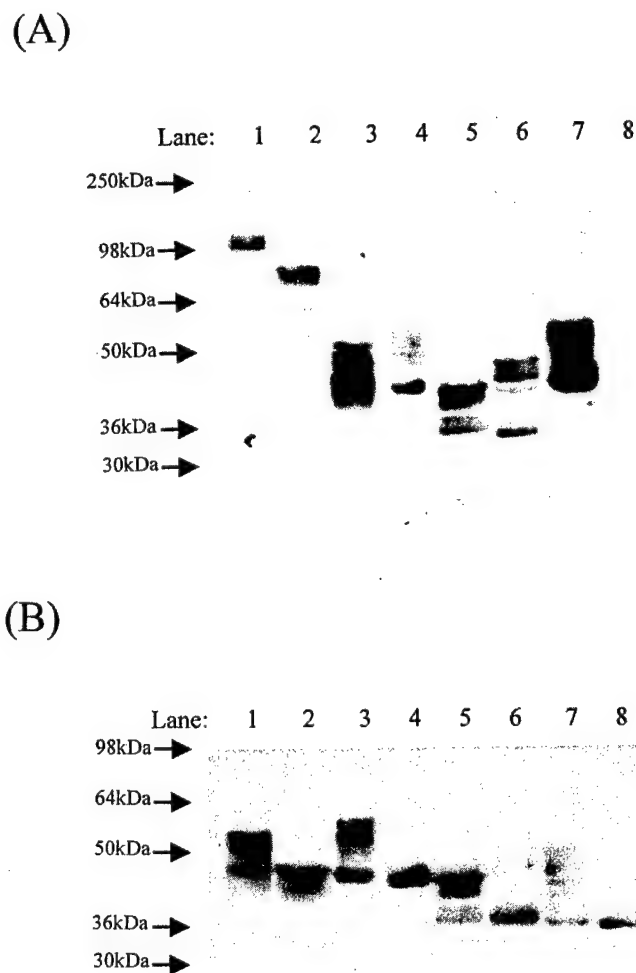


FIG. 3. Immunoblot analyses of Flt-1/embigin chimeric receptors. (A) Flt-1/embigin chimeric receptors were separated by SDS-PAGE, transferred to PVDF, and then immunoblotted with an anti-Flt-1 antibody. Lanes: 1, Flt-1 Δ TK; 2, F1234/12E; 3, F123/E; 4, F12/2E; 5, F23/E; 6, F2/2E; 7, F1/12E; 8, embigin. (B) High-5 cells expressing chimeric proteins were incubated in the presence of tunicamycin for 24 h prior to the preparation of membranes. Lanes: 1 and 2, F123/E; 3 and 4, F12/2E; 5 and 6, F23/E; 7 and 8, F2/2E. Lanes 2, 4, 6 and 8 were treated with methanol containing tunicamycin, and lanes 1, 3, 5, and 7 were treated with methanol alone.

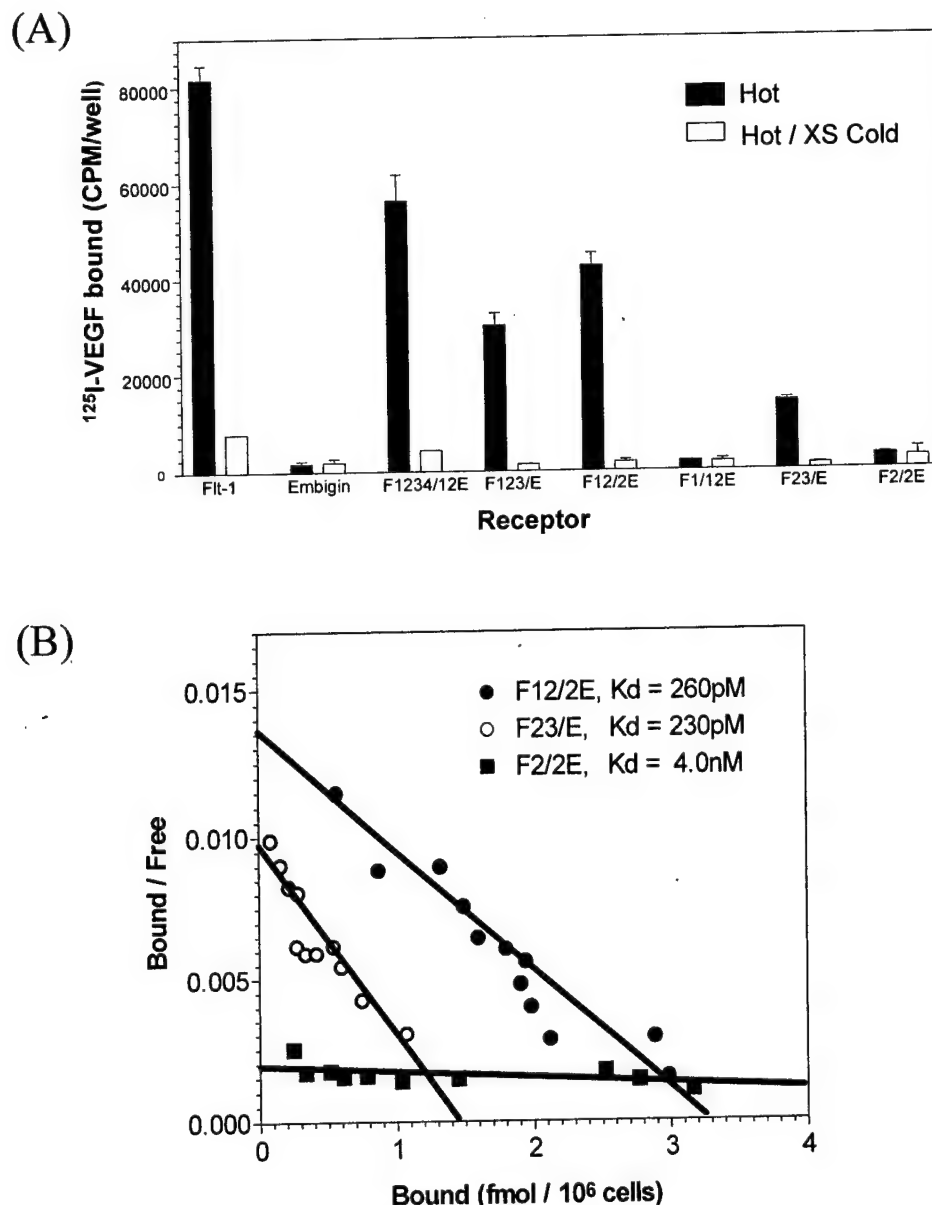


FIG. 4. VEGF binding to Flt-1/embigin chimeras. (A) 125 I-labeled VEGF was incubated with High-5 cells expressing chimeric receptors in the presence and absence of excess unlabeled VEGF. Error bars represent standard deviations ($n = 4$). (B) Comparison of Scatchard plots for the saturation binding of VEGF to the chimeric Flt-1/embigin receptors F12/2E, F23/E and F2/2E.

procedure cut Flt-1 loop-1 from F123/E cDNA resulting in the plasmid 1392-F23/E. **F2/2E:** the cDNA encoding Flt-1 loop-1 was excised from F12/2E chimera cDNA to make the chimera F2/2E, using the same strategy and primers used to make F23/E.

Flt-1 mutagenesis. Flt-1_{ΔTK} cDNA was mutagenized in the pVL-1392 plasmid using the QuickChange mutagenesis kit (Stratagene, Inc.). The R224A mutant was made using the oligonucleotides R224AF and R224AR, whereas the mutant D231A was made using the oligonucleotides D231AF and D231AR. All chimeric and mutant cDNA constructs were fully sequenced prior to use.

Recombinant protein expression. Insect cells were routinely grown in Grace's medium supplemented with 10% fetal bovine serum, 2% yeastolate, 2% lactalbumin hydrolysate and 10 mg/ml gentamicin. Recombinant cDNA was cotransfected into SF9 cells with

replication-defective Baculogold baculovirus DNA (PharMingen). Resulting baculoviruses were plaque-purified to generate pure baculoviral stocks, which were used to express recombinant proteins in High-Five insect cells. Recombinant human VEGF was purified from High-Five cell conditioned medium by heparin-Sepharose (Pharmacia) chromatography, followed by reverse-phase HPLC using a Vydac C₁₈ column (0.46 × 25 cm) (The Separations Group, Hesperia, CA) and molecular sieving HPLC on a Biosep-S3000 column (0.75 × 50 cm) (Phenomenex Inc., Torrance, CA). Flt-1, Flt-1_{ΔTK} and chimeric Flt-1/embigin receptors were expressed as membrane-bound proteins. High-Five cells expressing recombinant receptor proteins were sonicated in buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM PMSF, 50 mg/ml aprotinin and 50 mg/ml leupeptin (all from Sigma Chemical Co., St. Louis, MO), and membrane fragments

were collected by centrifugation at 228,000g. Protein glycosylation was inhibited by incubating cells with 0.5 mg/ml tunicamycin (Cal-Biochem, La Jolla, CA) from 24 h postinfection until membranes were harvested at 48 h postinfection.

VEGF binding assays. Recombinant VEGF was iodinated with ^{125}I to an average specific activity of 5.49×10^5 cpm/ng using the chloramine-T method (Montelaro and Rueckert, 1975). High-Five cells were seeded at 1×10^5 cells/well in 48-well plates and then infected with baculovirus encoding Flt-1, Flt-1/embigin chimeras or Flt-1 mutants. At 48 h post-infection, VEGF binding experiments were performed in a final volume of 100 μl /well. For VEGF binding tests, 100 pM [^{125}I]-VEGF was added to cells in the presence or absence of 100-fold excess unlabeled VEGF in binding buffer consisting of DME/F12 medium (pH 7.4) containing 5 mg/ml BSA (Sigma), 1 mg/ml heparin (Sigma) and 25 mM Hepes (Research Organics, Cleveland, OH). For saturation binding, 10 pM to 5 nM VEGF were added to cells in the presence or absence of 100-fold excess unlabeled VEGF. All binding reactions were incubated at 4°C for 8 h and then washed with PBS containing 5 mg/ml BSA. Cells were lysed with 0.1% NaOH, and bound radioactivity was quantified with an LKB Model 1261 gamma counter.

RESULTS

Biological activity of recombinant VEGF. Purified recombinant VEGF was quantified by amino acid analysis. Recombinant VEGF stimulated HUVEC proliferation in a time- and dose-dependent manner. In 5-day growth assays HUVEC numbers reached 2.8-fold control levels in the presence of 5 ng/ml VEGF (data not shown). The mitogenic effect of recombinant VEGF on HUVEC was maximal at a concentration of 10 ng/ml (Fig. 2A). Thus, recombinant VEGF expressed by insect cells was as potent a mitogen for HUVEC as natural VEGF produced by A431 cells (Myoken *et al.*, 1991).

Characterization of the minimal VEGF binding site of Flt-1. Recombinant Flt-1 $_{\Delta\text{TK}}$ expressed by insect cells was detected by western blotting as a 110 kDa membrane-bound protein using an N-terminal-specific anti-Flt-1 antibody (Fig. 2B). Flt-1 $_{\Delta\text{TK}}$ contained the seven extracellular Ig-loops and the transmembrane region of Flt-1, but it lacked the intracellular tyrosine kinase domain. Intact Flt-1 migrated as a 190 kDa band while recombinant embigin was not recognized by the anti-Flt-1 antibody. VEGF bound to Flt-1 $_{\Delta\text{TK}}$ in a saturable manner with an apparent K_d of 190 pM (Fig. 2C). Chimeric Flt-1/embigin receptors had lower molecular masses relative to Flt-1 $_{\Delta\text{TK}}$, reflecting fewer Ig-loops (Figs. 1 and 3A). Multiple bands were recognized for chimeric receptors containing three or less Ig-loops. To determine whether or not these multiple bands were due to differential receptor glycosylation cells transiently expressing F123/E, F12/2E, F23/E or F2/2E chimeras were incubated in the presence or absence of the glycosylation inhibitor tunicamycin (Fig. 3B). In the presence of tunicamycin, non-glycosylated chimeric receptors were detected as single bands, which indicated that the multiple bands detected for each chi-

TABLE 2
Affinity of VEGF Binding to Chimeric
and Mutant Flt-1 Receptors

Receptor	VEGF binding affinity (K_d value)	Relative K_d value
Flt-1	190 pM	1.00
F1234/12E	250 pM	1.32
F123/E	180 pM	0.95
F12/2E	260 pM	1.37
F23/E	230 pM	1.21
F2/2E	4000 pM	21.58
F1/12E	N.D.	N.D.
R224A	180 pM	0.95
D231A	130 pM	0.68

mera resulted from differential glycosylation of a single receptor protein.

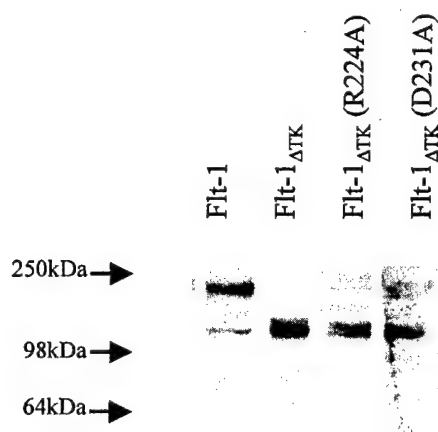
VEGF bound to Flt-1 and to most chimeric Flt-1/embigin receptors, but not to embigin (Fig. 4A). VEGF bound to chimeric receptors containing Flt-1 Ig-loops 1–4, 1–3, or 1–2 (F1234/12E, F123/E, and F12/2E, respectively) with affinities similar to that of Flt-1 $_{\Delta\text{TK}}$ containing an intact extracellular domain (Table 2). However, replacing Flt-1 loop-2 with an embigin Ig-loop (F1/12E) abolished VEGF binding. These results indicated that loop-2 of Flt-1 was required for VEGF binding, and they demonstrated the inability of Flt-1 loop-1 alone to support VEGF binding. Deleting Flt-1 loop-1 (F23/E) did not significantly reduce the affinity of VEGF binding (Fig. 4B). Despite the requirement for Flt-1 loop-2 for binding, a chimeric receptor containing only loop-2 of Flt-1 (F2/2E) bound VEGF very weakly.

Ability of Flt-1 mutants to bind VEGF. The Flt-1 $_{\Delta\text{TK}}$ receptor mutants R224A and D231A were expressed on the surface of insect cells (Fig. 5A), and the protein products had similar molecular masses as wild-type Flt-1 $_{\Delta\text{TK}}$. VEGF bound to the mutants R224A and D231A with affinities similar to membrane-bound Flt-1 $_{\Delta\text{TK}}$ ($K_d = 190$ pM) (Fig. 5B): VEGF bound to the R224A mutant with a K_d of 180 pM and to the D231A mutant with a K_d of 130 pM.

DISCUSSION

VEGF was previously reported to bind with high affinity to an Flt-1/Flt-4 chimeric receptor containing only loop-2 of Flt-1 (Davis-Smyth *et al.*, 1996). However, given that Flt-4 is homologous to Flt-1, chimeras between Flt-1 and a less closely related protein may have provided additional information about the minimal structural requirements of Flt-1 for VEGF binding. Other reports indicated that loops 1–2 or 1–3 of soluble Flt-1 receptors were required for high-affinity VEGF binding (Cunningham *et al.*, 1997b; Barleon *et al.*, 1997). Possible explanations for the disparity of

(A)



(B)

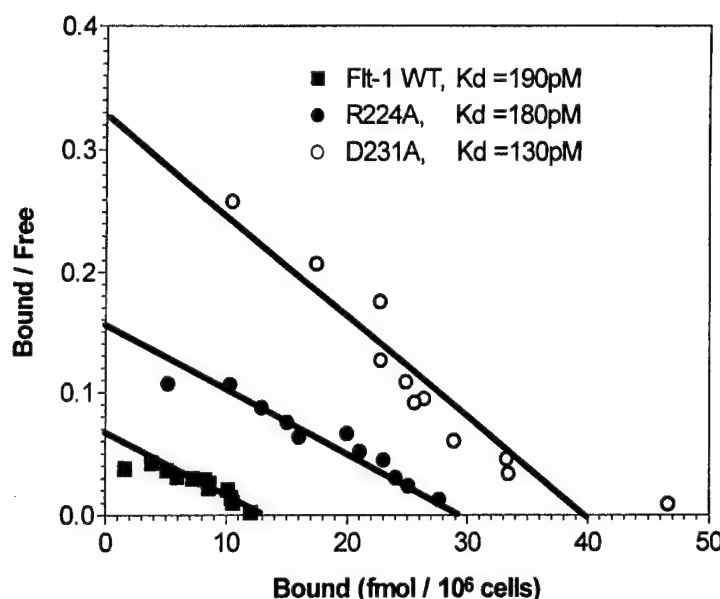


FIG. 5. Analyses of Flt-1 Δ TK mutants. (A) Wild-type and mutant Flt-1 Δ TK receptors were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with anti-Flt-1 polyclonal antibody. (B) Comparison of Scatchard plots for the saturation binding of VEGF to wild-type and mutant Flt-1 Δ TK receptors on intact High-5 cells.

these findings are differences in the experimental systems used, the domain boundaries chosen, the inclusion of non-Flt-1 Ig-loops, and the nature of non-Flt-1 flanking Ig-loops. The use of membrane-bound Flt-1/embigin chimeric receptors in our experimental system was chosen to reflect VEGF binding to endogenous membrane-bound Flt-1, while minimizing any potential contributions of non-Flt-1 sequences. We found that although Ig-loop 2 of Flt-1 played a dominant role in VEGF binding, it was not sufficient of itself for high

affinity binding. Strong VEGF binding required loop-2 of Flt-1 in combination with either loop-1 or loop-3. While individually deleting or replacing loops-1 or 3 of Flt-1 with embigin Ig-loops did not significantly affect the affinity of VEGF binding, simultaneously replacing or deleting both loops 1 and 3 of Flt-1 drastically affected binding (Table 2). Our results indicate that the Ig-loops surrounding loop-2 of Flt-1 do play an important role in binding VEGF and that although they can be substituted for by the homologous Ig-loops of Flt-4,

they cannot be functionally replaced by embigin Ig-loops.

Replacing the Flt-1 amino acid residues Arg-224 and Asp-231 with alanines did not reduce the affinity for VEGF in the context of a full-length membrane-bound Flt-1 extracellular domain (Fig. 5B and Table 2). This finding contrasts with a previous report that Asp-231 of Flt-1 was functionally required for full VEGF binding to soluble Flt-1₍₁₋₃₎ (Davis-Smyth *et al.*, 1998). The reason for these different results is unknown. However, it is plausible that the replacement of Asp at position 231 in a complete Flt-1 extracellular domain has less of an impact on ligand binding than it does in a truncated extracellular domain. Further mutational analysis of the extracellular domain of membrane-bound Flt-1 should provide insights into the contributions of specific Flt-1 amino acid residues and sequences to the ligand-binding activity of this receptor.

A recent report using soluble KDR-IgG receptors found that Ig-loop domains 2-3 of KDR were essential for VEGF binding (Fuh *et al.*, 1998). Thus, there are similarities between the location of the VEGF binding site of Flt-1 and KDR, with the exception that Ig-loop 3 of KDR appears to be strictly required for VEGF binding. Although there is increasing evidence that the mitogenic effect of VEGF is mediated by KDR, the importance of Flt-1 is emphasized by the lethal effect of disrupting this gene in mouse embryonic development (Fong *et al.*, 1995). In contrast, mice developed normal vessels and survived when the tyrosine kinase domain of Flt-1 was deleted (Flt-1^{TK-/-}) without affecting the ligand binding region (Hiratsuka *et al.*, 1998). However, these Flt-1^{TK-/-} mice had suppressed VEGF-dependent macrophage migration. This suggests that the major role of Flt-1 in blood vessel development may be as a ligand binding molecule rather than as a signaling molecule.

In conclusion, our finding that the minimal VEGF binding region of Flt-1 involves two of the three N-terminal Ig-loop domains of Flt-1 is reminiscent of the ligand binding determinants described for other Ig-loop-containing growth factor receptor tyrosine kinases including α PDGFR (Heidaran *et al.*, 1990), β PDGFR (Heidaran *et al.*, 1995), c-Kit (Lev *et al.*, 1993) and FGFRs 1 and 2 (Johnson *et al.*, 1990). This suggests that Ig-loop containing growth factor receptors bind their ligands through contiguous receptor domains that assemble conformationally into ligand binding sites.

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MAP Kinases, Phosphatidylinositol 3-Kinase, and p70 S6 Kinase Mediate the Mitogenic Response of Human Endothelial Cells to Vascular Endothelial Growth Factor

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Although the significance of vascular endothelial growth factor (VEGF) and its receptors in angiogenesis is well established, the signal transduction cascades activated by VEGF and their involvement in mediating the mitogenic response of endothelial cells to VEGF are incompletely characterized. Here we demonstrate that VEGF activates mitogen-activated protein (MAP) kinases, including the extracellular signal-regulated protein kinase (ERK) and p38 MAP kinase, phosphatidylinositol 3-kinase (PI 3-kinase), and p70 S6 kinase in human umbilical vein endothelial cells (HUVEC). The activation of these enzymes was assayed by kinase phosphorylation and by kinase activity towards substrates. Studies with PI 3-kinase inhibitors revealed that activation of p70 S6 kinase was mediated by PI 3-kinase. Selective inhibition of ERK, PI 3-kinase, and p70 S6 kinase with the inhibitors PD098059, LY294002, and rapamycin, respectively, inhibited VEGF-stimulated HUVEC proliferation. In marked contrast, the p38 MAP kinase inhibitor SB203580 not only failed to inhibit but actually enhanced HUVEC proliferation; this effect was associated with the phosphorylation of Rb protein. Rb phosphorylation resulted from a decrease in the level of the cdk inhibitor p27^{Kip1}. These results indicate that the activities of ERK, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation. p38 MAP kinase suppresses endothelial cell proliferation by regulating cell-cycle progression. *J Cell Physiol* 178:235-246, 1999. © 1999 Wiley-Liss, Inc.

Vascular endothelial growth factor (VEGF) is a key regulator in both physiological and pathological angiogenesis (Connolly et al., 1989; Kim et al., 1993; Carmeliet et al., 1996). VEGF is distinct from other angiogenic factors in that it is an endothelial cell-specific mitogen (Leung et al., 1989; Keck et al., 1989; Myoken et al., 1991). VEGF plays a critical role in the regulation of endothelial cell proliferation, which is a major step in angiogenesis (Risau, 1997). The biological effects of VEGF are mediated by the specific receptor tyrosine kinases KDR/Flk1 and Flt1, which are composed of seven extracellular Ig-like domains, a single transmembrane sequence, and an intracellular region containing split tyrosine kinases (Mathews et al., 1991; de Vries et al., 1992; Terman et al., 1992; Quinn et al., 1993). Although it is known that VEGF receptors undergo autophosphorylation in response to VEGF (Myoken et al., 1991; Quinn et al., 1993; Seetharam et al., 1995), the subsequent biochemical events mediating the mitogenic response of endothelial cells to VEGF have not been completely characterized. Previous studies have demonstrated the VEGF-dependent phosphorylation of several cytoplasmic signaling proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), PLC γ , Ras GTPase-activating protein, the adapter proteins NcK (Guo et al., 1995) and Shc (Kroll and Walt-

enberger, 1997), and focal adhesion kinase (Abedi and Zachary, 1997). These proteins can potentially participate in receptor signaling pathways.

In mammalian cells, ligand binding to receptor tyrosine kinases trigger the activation of downstream signaling enzymes, including MAP kinase, PI 3-kinase, p70 S6 kinase, and PLC γ (Marshall, 1995). Activation of these signaling intermediates transduces extracellular signals to the nucleus and ultimately regulates gene expression and cellular responses such as cell proliferation, migration, differentiation, and apoptosis. This model has not been completely tested with respect to VEGF-receptor signaling. The goal of the present study was to characterize the signaling enzymes that mediated the mitogenic response of endothelial cells to

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VEGF, ERK, p38 MAP kinase, PI 3-kinase, and p70 S6 kinase were studied.

Three distinct MAP kinases, ERK, p38 MAP kinase, and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, have been shown to respond to growth factors, stress, and inflammatory cytokines (Cobb and Goldsmith, 1995; Raingeaud et al., 1995). ERK, which consists of two members, ERK1 and ERK2, phosphorylates a variety of intracellular proteins, including other kinases and transcription factors (Hill and Treisman, 1995). Signaling through the well-characterized Ras/Raf/MEK/ERK cascade can regulate differentiation, proliferation, or oncogenic transformation depending on cellular context (Marshall, 1995). JNK phosphorylates and activates the transcription factors c-jun and activating transcription factor-2 (ATF-2) (Pulverer et al., 1991; Gupta et al., 1995). A complex of c-jun and ATF-2 binds to the AP-1 site of the c-jun promoter, thereby stimulating its transcription. p38 MAP kinase is a potent activator of MAPKAP kinase-2, which phosphorylates the small heat shock protein hsp27 (Freshney et al., 1994; Rouse et al., 1994). Hsp27 in turn has been suggested to modulate actin microfilament dynamics and cellular thermoresistance (Lavoie et al., 1995). Activation of PI 3-kinase, which catalyzes the formation of phosphatidylinositol 3, 4, 5-phosphate (PIP3), has been implicated in the regulation of many cellular processes, including actin cytoskeleton reorganization and cell proliferation and migration (Fantl et al., 1992; Kundra et al., 1994; Wennström et al., 1994). p70 S6 kinase is a mitogen-regulated ser/thr kinase consisting of cytosolic p70 and nuclear p85 isoforms. This enzyme phosphorylates 40S ribosomal protein S6, which itself plays a role in protein synthesis (Erikson, 1991; Kozma and Thomas, 1994). VEGF-stimulated ERK and p38 MAP kinase enzymatic activities have been described by others (D'Angelo et al., 1995; Seetharam et al., 1995; Rousseau et al., 1997). However, the individual roles of ERK and p38 MAP kinase in the regulation of VEGF-induced endothelial cell proliferation were not elucidated. The presence, activation, and function of PI 3-kinase and p70 S6 kinase in human endothelial cells have not been previously explored.

In this study, we have thoroughly characterized VEGF-stimulated activation of ERK, p38 MAP kinase, PI 3-kinase, and p70 S6 kinase in human umbilical vein endothelial cells (HUVEC). These cells endogenously express KDR and Flt1 VEGF receptors (Waltenberger et al., 1994) and not supraphysiological levels of recombinant VEGF receptors. With potent inhibitors of MEK, p38 MAP kinase, PI 3-kinase, and p70 S6 kinase, our data indicate that the activities of MAP kinase, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced endothelial cell proliferation, while p38 MAP kinase has a negative effect on endothelial cell growth, and its activity is associated with suppression of Rb phosphorylation.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (F-12) were purchased from Life Technologies, Inc. (Bethesda, MD). Fetal bovine serum, type I collagen, basic fibroblast growth factor (bFGF),

and EGF were from Upstate Biotechnology (Lake Placid, NY). DiI-Ac-LDL was from Biomedical Technologies (Stoughton, MA). Antibodies used were as follows: polyclonal anti-phospho-ERK, anti-phospho-p38 MAP kinase, and anti-p38 MAP kinase antibodies from New England Biolabs (Beverly, MA); polyclonal anti-MAP kinase R2 (Erk1-CT), monoclonal anti-ERK2, and polyclonal anti-MAPKAP kinase-2 from Upstate Biotechnology; polyclonal anti-p70 S6 kinase and anti-cyclin D1 from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-Rb from Pharmingen (San Diego, CA); and monoclonal anti-PI 3-kinase (p85) and anti-phosphotyrosine (PY20) from Transduction Laboratories (Lexington, KY). Inhibitors used were PD098059 from Research Biochemicals International (Natick, MA), LY294002 from Biomol (Plymouth Meeting, PA), rapamycin from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), and SB203580 from Calbiochem (La Jolla, CA). Kinase substrates used were myelin basic protein (MBP) and MAPKAP kinase-2 substrate peptide (KKLNRTLSSVA) from Upstate Biotechnology, p70 S6 kinase substrate peptide (RRLSSLRA) from Santa Cruz Biotechnology, and phosphatidylinositol from Sigma (St. Louis, MO). Protein A-Sepharose and protein G-agarose were from Pierce (Rockford, IL). [γ - 32 P] ATP was from New England Nuclear (Boston, MA). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham (Arlington Heights, IL). Silica gel 60 TLC plates were from EM-Science (Fort Washington, PA). All other chemicals were from Sigma.

VEGF

Human VEGF₁₆₅ cDNA was cloned from A431 epidermoid carcinoma cells by RT-PCR and subcloned into the baculovirus expression vector PVL1392 (Invitrogen, Carlsbad, CA). After infection of High 5 insect cells (Invitrogen) with recombinant virus encoding VEGF, VEGF was purified from conditioned medium by heparin-Sepharose chromatography, followed by reverse phase HPLC and gel filtration HPLC. The concentration of VEGF was determined by amino acid analysis, and its identity was confirmed by N-terminal amino acid microsequencing. The mitogenic activity of recombinant VEGF was tested on HUVEC.

Cell culture and cell proliferation assay

HUVEC were isolated from human umbilical cord veins as described by Hoshi and McKeehan (1984) and maintained in collagen-coated tissue culture plates in complete growth medium (DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 10 ng/ml bFGF, 10 μ g/ml heparin, and 100 μ g/ml kanamycin) at 37°C in 5% CO₂. HUVEC were identified as endothelial cells by their uptake of fluorescent acetylated low density lipoprotein (Voyta et al., 1984). HUVEC from passages 6–12 were used in this study. Confluent cell cultures were dispersed and plated at a density of 2×10^4 cells/cm² in culture plates (80% confluent) in complete growth medium or DMEM/F-12 containing 10% FBS. Five to eight hours later, the cells were serum-starved in DMEM/F-12 containing 0.1–1% FBS for 15–20 h. Prior to the addition of growth factors or inhibitors, the medium was replaced with fresh DMEM/F-12. After

serum starvation, no significant apoptosis of the adherent cells was detected by flow cytometry or TUNEL assay. In cell proliferation assays, HUVEC were seeded at a density of 1×10^4 cells per well into 24-well tissue culture plates in DMEM/F-12 containing 10% FBS; 12 h later the indicated reagents were added. After 5 days, cell numbers were counted with a Coulter (Hialeah, FL) counter. Cell viability was assessed by the ability of the cells to exclude trypan blue.

ERK2 and p70 S6 kinase mobility shift assays

Equal numbers of untreated or growth factor-stimulated cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in $2\times$ SDS-PAGE sample buffer (Laemmli, 1970). After boiling at 100°C for 5 min, the lysates were briefly sonicated and then subjected to SDS-PAGE using the following polyacrylamide gel formulae: 15% acrylamide/0.075% bisacrylamide for ERK2 and 7.5% acrylamide/0.075% bisacrylamide for p70 S6 kinase. Separated proteins were transferred to PVDF membranes and analyzed by immunoblotting with antibodies to ERK2 and p70 S6 kinase.

Immunoprecipitation of kinase activity

After treatment, the cells were lysed in ERK lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM β -glycerophosphate, 0.25% sodium deoxycholate, 1% Triton X-100), MAPKAP kinase-2 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 20 mM NaF, 2 mM sodium pyrophosphate, 1% Triton X-100), PI 3-kinase lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40), or p70 S6 kinase lysis buffer (10 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl_2 , 2 mM DTT, 0.5% NP-40) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ pepstatin. The lysates were cleared by centrifugation at 13,000g for 15 min at 4°C , and anti-MAP kinase R2, anti-MAPKAP kinase-2, anti-phosphotyrosine PY20, or anti-p70 S6 kinase antibodies were added. After 1 h incubation at 4°C , 30 μl of a 50% protein A-Sepharose slurry were added, and the lysates were rotated for 1 h at 4°C .

In vitro ERK assay

ERK activity was assessed by performing immune complex kinase assays (Pages et al., 1994). Briefly, ERK immunoprecipitates were washed twice in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1% NP-40) and once in kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl_2 , 1 mM MnCl_2 , 10 mM p -nitrophenylphosphate, 0.1 mM Na_3VO_4 , 1 mM DTT). The immune complexes were incubated at 30°C for 20 min with 30 μl of kinase buffer containing 8 μg MBP, 20 μM ATP, and 5 μCi [γ - ^{32}P] ATP. The assay was terminated by the addition of $6\times$ SDS-PAGE sample buffer. The products were resolved on 15% SDS-PAGE and transferred to PVDF membranes. Phosphorylated MBP was visualized by autoradiography. ERK was quantified by Western blot analysis.

Kinase assay of MAPKAP kinase-2

Kinase activity was measured as previously described by Foltz et al. (1997), Clifton et al. (1996), and Crawley et al. (1996) with the following modifications. The immunoprecipitates were washed three times in lysis buffer and twice in kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl_2 , 25 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 2 mM DTT, and 25 μM ATP). The kinase assay was initiated by the addition of 25 μl kinase buffer containing 1 μg of peptide substrate (KKLN-RTLSVA) and 10 μCi of [γ - ^{32}P] ATP. After 20 min at 30°C , the reaction was terminated by adding $6\times$ SDS-PAGE sample buffer. The phosphorylated peptide substrate was resolved by SDS-PAGE in 20% acrylamide/0.07% bisacrylamide in the presence of 0.72 M β -mercaptoethanol, and it was visualized by autoradiography of the dried SDS-polyacrylamide gel.

In vitro PI 3-kinase assay

Lipid kinase assays were performed as described by Whitman et al. (1985) with the following modifications. After washing three times in lysis buffer and once in kinase buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA), the immunoprecipitates were incubated with 10 μg of phosphatidylinositol sonicated in 50 μl of kinase buffer at 25°C for 10 min. One microliter of 1 M MgCl_2 and 10 μCi [γ - ^{32}P] ATP were added, and the incubation was continued for a further 10 min. Reactions were stopped by the addition of 0.1 ml of 1 N HCl and 0.2 ml chloroform/methanol (1:1). The organic phase was washed once with methanol/1 N HCl (1:1). Phospholipids were separated on potassium oxalate-treated thin layer chromatography (TLC) plates and developed with chloroform/methanol/30% ammonium hydroxide/ H_2O (46: 41:5:8). The TLC plates were dried and subjected to autoradiography. Quantitation of radioactivity incorporated into lipids was performed by liquid scintillation counting of radioactive spots.

In vitro p70 S6 kinase assay

The assay was carried out as described by Crawley et al. (1996) and Dahl et al. (1996). After washing, the immunoprecipitates were resuspended in 10 μl of kinase buffer (50 mM MOPS, pH 7.2, 1 mM DTT, 30 μM ATP, 5 mM MgCl_2 , 10 mM p -nitrophenylphosphate) containing 0.5 μg S6 peptide substrate (RRRLSSLRA), 10 ng IP-20 (protein kinase A inhibitor) and 5 μCi [γ - ^{32}P] ATP. Reactions proceeded for 30 min at 30°C , and they were stopped by the addition of $6\times$ SDS-PAGE sample buffer. The phosphorylated peptide substrate was resolved by SDS-PAGE in 20% acrylamide/0.07% bisacrylamide in the presence of 0.72 M β -mercaptoethanol and visualized by autoradiography. The radioactive bands were excised from the gel and quantified by liquid scintillation counting.

RESULTS

VEGF stimulates the activation of ERK in HUVEC

To determine whether VEGF induced phosphorylation of ERK, we treated serum-starved HUVEC with 50 ng/ml VEGF for the indicated times (Fig. 1A). Western blot analysis of cell lysates demonstrated that ERK2 was transiently phosphorylated. Phosphorylated ERK2

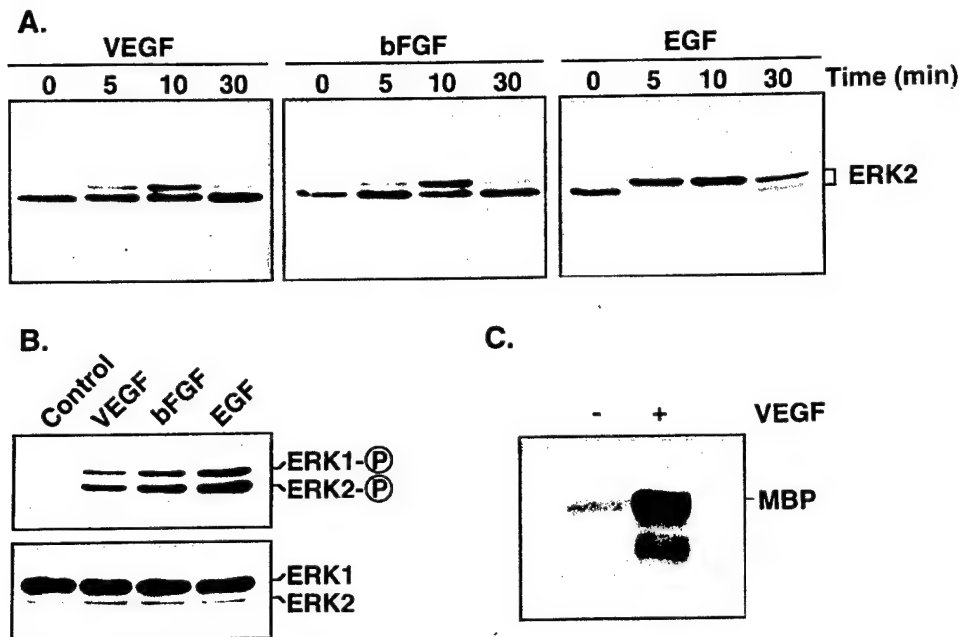


Fig. 1. VEGF-stimulated ERK activation in HUVEC. **A:** VEGF-stimulated ERK2 electrophoretic mobility shift. Serum-starved cells were left untreated or stimulated with VEGF (50 ng/ml), bFGF (50 ng/ml) or EGF (20 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting with an anti-ERK2 monoclonal antibody. **B:** VEGF-stimulated phosphorylation of ERK1 and ERK2. Cells were left untreated (Control) or stimulated with VEGF, bFGF or EGF for 10 min. Phosphorylated ERK1 and ERK2 were detected with phos-

phospecific ERK antibodies (upper panel). After being stripped, the blot was reprobed with anti-ERK antibodies to quantitate loading (lower panel). **C:** VEGF-stimulated ERK activity. Cells were left untreated (-) or stimulated with VEGF (50 ng/ml) (+) for 10 min. Kinase activity of ERK was determined against MBP in an *in vitro* kinase assay. The results shown are representative of three independent experiments.

exhibited reduced electrophoretic mobility relative to the nonphosphorylated form. Phosphorylation was detectable within 5 min, reached a peak within 10 min, and decreased to near basal level by 30 min. The pattern of ERK2 phosphorylation in control cells treated with 50 ng/ml bFGF was similar to that induced by VEGF, while the cells exposed to 20 ng/ml EGF showed a faster and stronger response. The kinetics of ERK1 phosphorylation in response to VEGF was similar to that of ERK2 (data not shown). VEGF-induced phosphorylation of both ERK1 and ERK2 was also observed in Western blots probed with specific antibodies to phosphorylated ERK (Fig. 1B). In dose-response experiments, 10 ng/ml VEGF, which is sufficient for optimal HUVEC proliferation *in vitro*, induced the same level of ERK phosphorylation as 50 ng/ml VEGF (data not shown). ERK activation was further confirmed in an enzymatic activity assay of ERK immunoprecipitates using MBP as substrates (Fig. 1C). Treatment of cells with VEGF for 10 min resulted in an eightfold increase in MBP phosphorylation. These results clearly indicated that VEGF activated ERK in HUVEC.

Activation of p38 MAP kinase by VEGF in HUVEC

To determine the kinetics of VEGF-induced p38 MAP kinase phosphorylation, we used an antibody specific for active p38 MAP kinase. This antibody recognizes p38 MAP kinase phosphorylated on the threonine and tyrosine of the TGY motif (Han et al., 1994; Raingeaud et al., 1995). Stimulation of HUVEC with VEGF re-

sulted in a rapid and transient phosphorylation of p38 MAP kinase, with a maximal response at 10 min (Fig. 2A). In contrast, no VEGF-dependent phosphorylation of the p54 and p46 isoforms of c-jun N-terminal kinase (JNK) was observed (data not shown). To demonstrate that VEGF stimulated p38 MAP kinase activity in these cells, we evaluated the activation of MAPKAP kinase-2, which is an *in vivo* substrate of p38 MAP kinase (Freshney et al., 1994; Rouse et al., 1994). MAPKAP kinase-2 was immunoprecipitated from cells treated with either VEGF or control tumor necrosis factor (TNF) α (Pietersma et al., 1997), and its activity was measured in an *in vitro* kinase assay (Fig. 2B). VEGF induced the activation of MAPKAP kinase-2 in a time-dependent manner. Maximal activity was observed at 10 min. Similar results were obtained when the kinase assay was performed using recombinant small heat shock protein (Hsp27) as a substrate (data not shown). To examine whether the activation of MAPKAP kinase-2 was due to p38 MAP kinase, we pretreated cells with SB203580, a specific inhibitor of p38 MAP kinase (Young et al., 1997), prior to VEGF addition. Pretreatment with 10 μ M SB203580 for 20 min blocked the VEGF-induced activation of MAPKAP kinase-2, indicating MAPKAP kinase-2 is downstream of p38 MAP kinase in HUVEC (Fig. 2B).

VEGF induces PI 3-kinase activation in HUVEC

To test for a possible role of PI 3-kinase as a signaling intermediate for VEGF receptors in HUVEC, we determined whether VEGF stimulated PI 3-kinase activity.

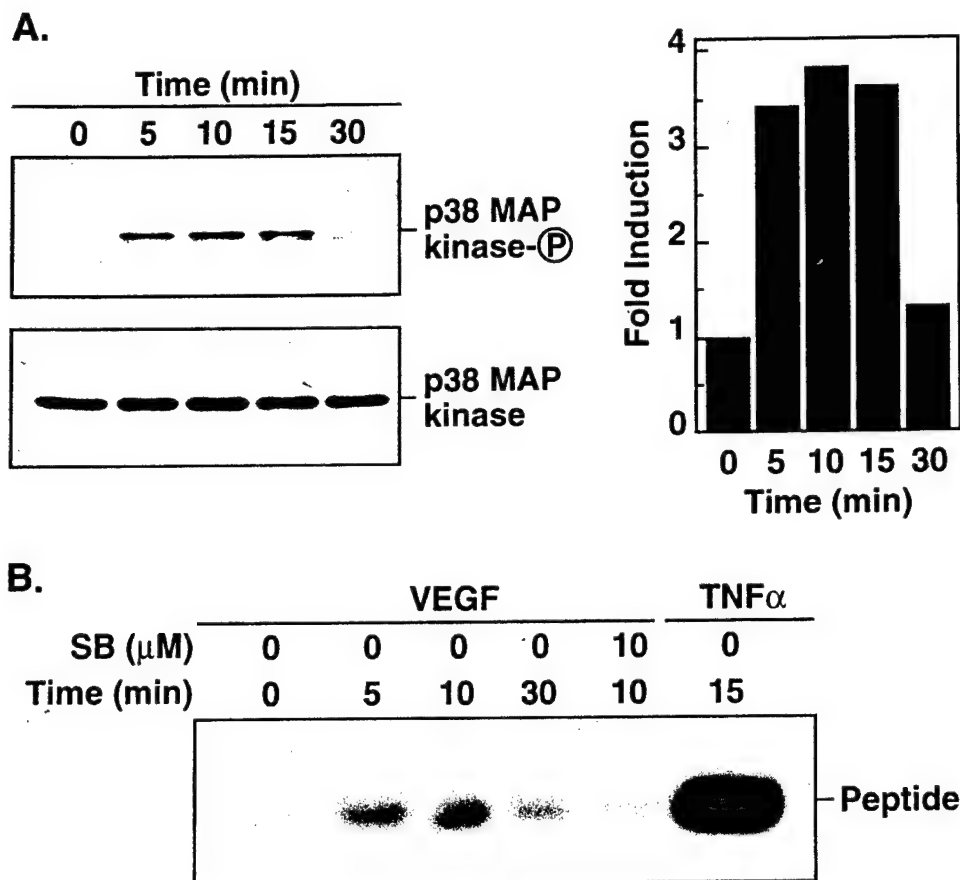


Fig. 2. VEGF-stimulated p38 MAP kinase activation in HUVEC. **A:** Phosphorylation of p38 MAP kinase by VEGF. Serum-starved cells were left untreated or were stimulated with VEGF (50 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting with a phosphospecific p38 MAP kinase antibody (left upper panel). The blot was reprobed with an anti-p38 MAP kinase antibody (left lower panel). Phosphorylation of p38 MAP kinase was quantitated by densitometry (right panel). The results shown are representative of

three independent experiments. **B:** Activation of MAPKAP kinase-2 by VEGF. After incubation with or without 10 μM of SB203580 (SB) for 20 min, the cells were then left untreated or treated with VEGF (50 ng/ml) or TNF α (50 ng/ml) as a positive control for the indicated times. Activity of MAPKAP kinase-2 was determined towards a synthetic peptide substrate (KKLNRTLVA). The phosphorylated peptide was visualized after SDS-PAGE by autoradiography. The results shown are representative of two independent experiments.

Kinase activity was assessed by anti-phosphotyrosine immunoprecipitation followed by *in vitro* kinase assays using phosphatidylinositol as a substrate. VEGF stimulated a transient burst of lipid kinase activity. The increase was clearly detectable by 2 min and was maximal by 5 min (Fig. 3A,B). Maximal activation was three to four times the untreated control. The increased kinase activity was not due to variations in the amount of PI 3-kinase subjected to immunoprecipitation, as demonstrated by Western blot analysis (Fig. 3C).

VEGF stimulates p70 S6 kinase activation in HUVEC

In response to VEGF, both isoforms (p70 and p85) of p70 S6 kinase in HUVEC were hyperphosphorylated within 15 min, and they remained phosphorylated with a slight decrease for at least 60 min (Fig. 4A). The hyperphosphorylation resulted in reduced electrophoretic mobility of several bands due to phosphorylation of multiple sites on p70 S6 kinase (Chou and Blenis, 1996). EGF-induced phosphorylation of the ki-

nase decreased visibly by 45 min, which was consistent with the EGF effect on p70 S6 kinase activation in Swiss 3T3 cells (Chen et al., 1991). To determine whether VEGF-stimulated phosphorylation correlated with increased kinase activity, we exposed HUVEC to 50 ng/ml VEGF for various times. Kinase activity towards a synthetic peptide substrate was measured in p70 S6 kinase immune complexes. VEGF maximally induced p70 S6 kinase activity approximately fourfold over the untreated control, and the activity was sustained for at least 60 min (Fig. 4B). Thus, the kinetics of VEGF-stimulated p70 S6 kinase activity correlated well with that of the kinase phosphorylation induced by VEGF.

Interactions of ERK, PI 3-kinase, and p70 S6 kinase in VEGF-induced signal transduction in HUVEC

We used specific kinase inhibitors to investigate the possibility of cross-talk between the pathways activated by VEGF in HUVEC. PD098059 is a specific inhibitor for MEK (ERK kinase) (Alessi et al., 1995).

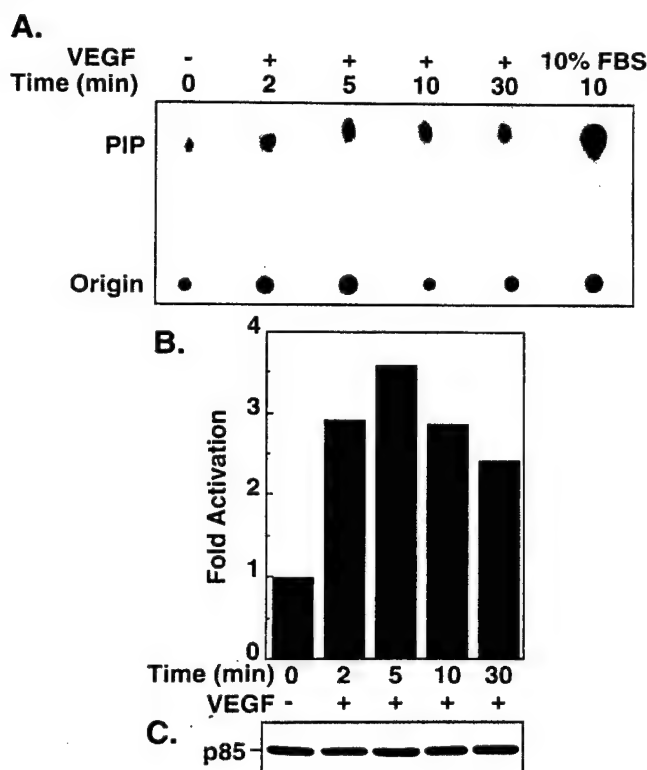


Fig. 3. VEGF-stimulated PI 3-kinase activation in HUVEC. Serum-starved cells were treated with VEGF (50 ng/ml) or 10% FBS as a positive control for the indicated times. Cell lysates were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody (PY20). The associated PI 3-kinase activity was assayed in the immune complexes against phosphatidylinositol (PI). **A:** Radioactive lipids were separated by thin layer chromatography and visualized by autoradiography. The positions of the origin and PI phosphate (PIP) are indicated. **B:** The radioactive spots were quantitated by liquid scintillation counting. Kinase activation is expressed as fold increase over the activity level at 0 min. **C:** PI 3-kinase in cell lysates used in the immunoprecipitations was quantitated by Western blotting with an anti-PI 3-kinase (p85) antibody. The results are representative of two independent experiments.

LY294002 and the immunosuppressant rapamycin have been characterized as selective inhibitors for PI 3-kinase and p70 S6 kinase, respectively (Chung et al., 1992; Vlahos et al., 1994). Serum-starved HUVEC were pretreated with either carrier (ethanol) or 10 nM rapamycin, 50 μ M LY294002, and 10 μ M PD098059 for 10 min, and 50 ng/ml VEGF or 20 ng/ml EGF were then added for the indicated times (Fig. 5, left panel). First we examined the roles of PI 3-kinase and p70 S6 kinase in VEGF-induced ERK activation. PD098059 completely blocked VEGF-stimulated phosphorylation of ERK2, indicating ERK was indeed downstream of MEK in these human endothelial cells. Pretreatment of cells with rapamycin or LY294002 failed to inhibit the mobility shift of ERK2 in response to VEGF. We then evaluated the roles of PI 3-kinase in VEGF-stimulated activation of p70 S6 kinase (Fig. 5, right panel). VEGF alone stimulated p70 S6 kinase hyperphosphorylation. Pretreatment of cells with rapamycin or LY294002 prevented the VEGF-induced phosphorylation of p70 S6 kinase. Both inhibitors reduced the basal phosphorylation level of this kinase, which was probably regulated

by a yet unknown phosphatase (Dennis et al., 1996; Pullen et al., 1998). Wortmannin, an LY294002 structurally unrelated inhibitor of PI 3-kinase (Ui et al., 1995), also inhibited phosphorylation of p70 S6 kinase by VEGF to basal levels. Taken together, these results indicate that PI 3-kinase is an upstream mediator of p70 S6 kinase activation and that neither PI 3-kinase nor p70 S6 kinase regulates VEGF-stimulated ERK activation. Similarly, MEK and ERK do not impinge on p70 S6 kinase, as demonstrated by the lack of effect of PD098059 on VEGF-stimulated phosphorylation of p70 S6 kinase (data not shown). Since Ras can directly bind PI 3-kinase in vitro (Rodriguez-Viciana et al., 1994), we cannot exclude the possibility of cross-talk between the ERK pathway and the PI 3-kinase pathway at the level of Ras.

ERK, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation

Since VEGF activated ERK, PI 3-kinase, and p70 S6 kinase, we examined their roles in mediating the VEGF-induced mitogenic response of endothelial cells. HUVEC were incubated in medium containing 10 ng/ml VEGF and increasing concentrations of inhibitors. In the absence of inhibitors, cell numbers increased two- to threefold in 5 days in response to VEGF stimulation (Fig. 6A). PD098059 and LY294002 inhibited cell proliferation in a dose-dependent manner and had IC_{50} s of ~ 5 μ M and ~ 450 nM, respectively. HUVEC were extremely sensitive to rapamycin, which had an IC_{50} of less than 10 pM. The three inhibitors individually completely blocked cell proliferation, while cell viability remained greater than 95%, as demonstrated by trypan blue exclusion. At concentrations that completely abolished VEGF-stimulated cell proliferation, the inhibitors had little effect on cell number in the absence of VEGF (Fig. 6C). Thus, the inhibitors were not intrinsically cytotoxic at the concentrations used, and their inhibitory effects on VEGF-induced HUVEC proliferation did not result from nonspecific cytotoxicity. These studies with specific inhibitors indicate that the activities of ERK, PI 3-kinase, and p70 S6 kinase are required for VEGF-induced mitogenesis, and they are not functionally interchangeable.

Role of p38 MAP kinase in HUVEC proliferation

To our knowledge, the role of the recently identified p38 MAP kinase in growth factor-induced cell proliferation has not been addressed. To investigate this issue, HUVEC were incubated with the specific p38 MAP kinase inhibitor SB203580 at a range of concentrations in the presence or absence of VEGF (Fig. 6B,C). Surprisingly, SB203580 not only failed to inhibit but strongly enhanced VEGF-induced cell proliferation in a dose-dependent manner (Fig. 6B). Moreover, SB203580 stimulated cell growth in the absence of VEGF. SB203580 at a concentration (10 μ M) that completely inhibited VEGF-induced p38 MAP kinase activity (Fig. 2) stimulated cell proliferation as well as 10 ng/ml VEGF alone. These data suggest that endogenous and VEGF-stimulated p38 MAP kinase activity suppresses endothelial cell proliferation.

To address the underlying mechanism by which p38 MAP kinase functions in endothelial cell proliferation,

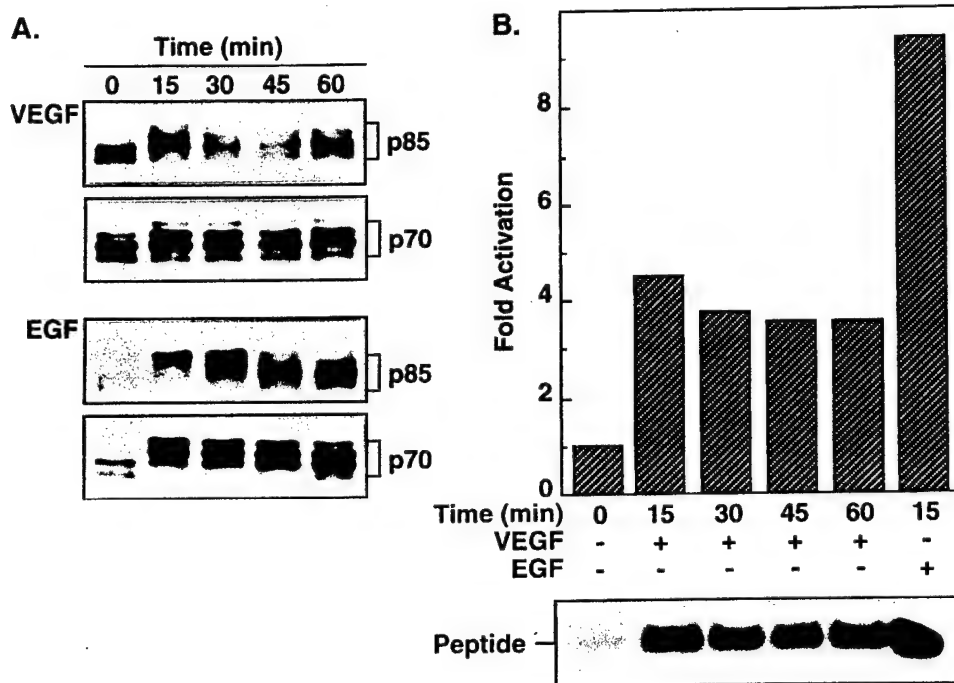


Fig. 4. VEGF-stimulated p70 S6 kinase activation in HUVEC. **A:** VEGF-stimulated p70 S6 kinase phosphorylation. Serum-starved cells were treated with VEGF (50 ng/ml) or control EGF (20 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting with anti-p70 S6 kinase antibodies. **B:** VEGF-stimulated p70 S6 kinase activity. p70 S6 kinase was immunoprecipitated from cells treated with VEGF (50 ng/ml) or control EGF (20 ng/ml) for the

indicated times. Kinase activity was assayed against a peptide substrate (RRRLSSLRA). Phosphorylated substrate was resolved on SDS-PAGE and visualized by autoradiography (**lower panel**). The peptide bands excised from the dried gel were quantitated by liquid scintillation counting (**upper panel**). The kinase activation is expressed as fold increase over the activity level at 0 min. The results shown are representative of three independent experiments.

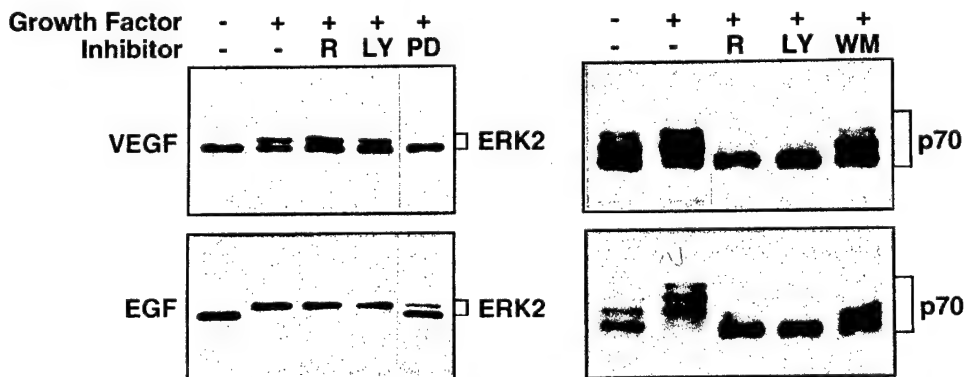


Fig. 5. Effects of rapamycin, LY294002, and PD098059 on VEGF-stimulated ERK and p70 S6 kinase activation in HUVEC. Serum-starved cells were preincubated with 0.1% ethanol (carrier), 10 nM rapamycin (R), 50 μ M LY294002 (LY), 100 nM wortmannin (WM) or 10 μ M PD098059 (PD) for 10 min. The cells were then left untreated (-) or stimulated (+) with VEGF (50 ng/ml) or EGF (20 ng/ml) for 10

min (**left panel**) or 20 min (**right panel**). The phosphorylation of ERK2 and p70 S6 kinase was detected by a shift in electrophoretic mobility with anti-ERK (left panel) and anti-p70 S6 kinase antibodies (right panel). The results shown are representative of three independent experiments.

we determined whether inhibition of p38 MAP kinase with SB203580 had any effect on early signaling events. Recently Zhang et al. (1997) reported that p38 MAP kinase had a negative effect on activation of ERK2 in mediating TNF α and arachidonic acid production in mast cells, suggesting communication between ERK and p38 MAP kinase. We therefore examined the effect of SB203580 on the activation of ERK. HUVEC

were incubated with either carrier (ethanol) or SB203580 for 20 min prior to stimulation with VEGF for 10 min (Fig. 7, upper panel). Pretreatment of the cells with SB203580 had no effect on VEGF-stimulated ERK phosphorylation. In addition, direct treatment of cells with SB203580 for 10 min did not activate ERK (Fig. 7, lower panel). We also observed no effect of this inhibitor on ERK activation in time-course experi-

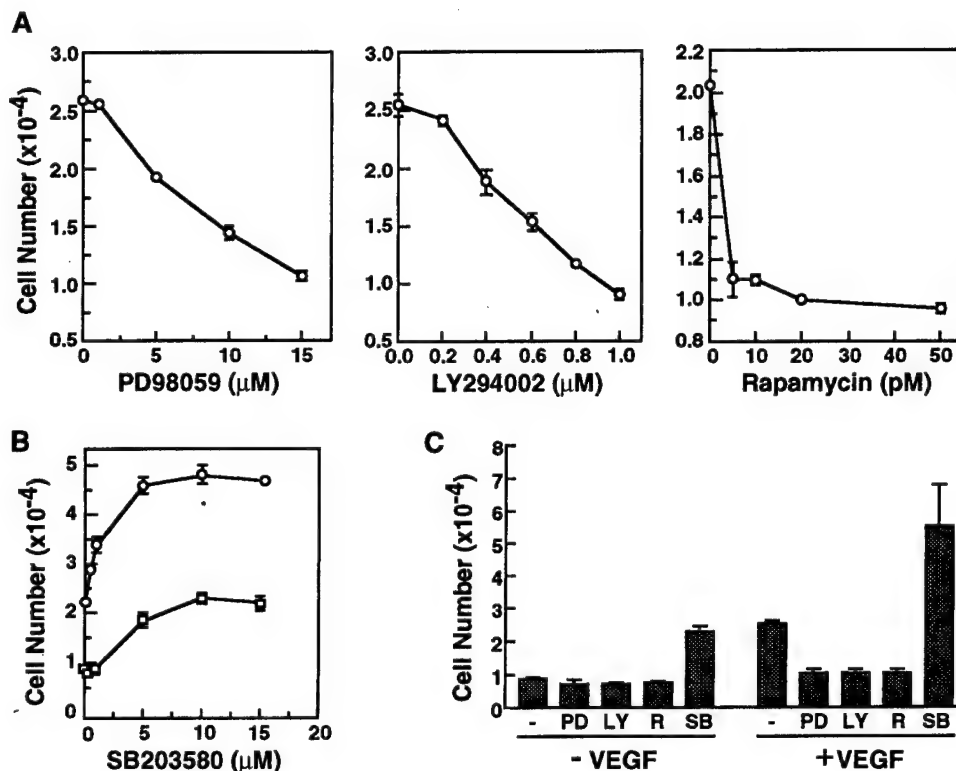


Fig. 6. Inhibitory effects of PD098059, rapamycin, LY294002, and SB203580 on VEGF-stimulated HUVEC proliferation. **A:** HUVEC (1×10^4 /well) in 24-well plates were incubated with 10 ng/ml VEGF and increasing concentrations of PD098059, rapamycin, or LY294002. Cell numbers were counted after 5 days. The results are presented as the mean \pm range of duplicate values and are representative of three independent experiments. **B:** HUVEC (1×10^4 /well) were incubated with the indicated concentrations of SB203580 in the absence (\square) and

presence (\circ) of 10 ng/ml VEGF for 5 days. The results are presented as the mean \pm range of duplicate values and are representative of four independent experiments. **C:** In the absence or presence of 10 ng/ml of VEGF, HUVEC (1×10^4 /well) were incubated with 15 μ M PD098059 (PD), 1 μ M LY294002 (LY), 20 pM rapamycin (R), or 10 μ M SB203580 (SB) for 4 days. The results are presented as the mean \pm SE of triplicate determinations and are representative of two independent experiments.

ments (data not shown). These results indicated that ERK was not a target of SB203580 action in these cells.

Intracellular signaling pathways transduce mitogenic signals to the nucleus leading to gene expression and subsequent cell-cycle progression. The hyperphosphorylation of retinoblastoma protein Rb by G1 cyclin-dependent kinases (cdks) in response to growth stimuli is a key event in S phase entry of the cell cycle (Sherr, 1994). Thus, phosphorylation of Rb is a useful indicator for cells that are entering the cell cycle. Since SB203580 stimulated cell proliferation, we examined its effects on the phosphorylation of Rb. HUVEC were rendered quiescent, and then they were treated for 24 h with SB203580, VEGF, VEGF and SB203580, or complete growth medium (Fig. 8). Western blot analysis showed that Rb in control cells incubated with 1% FBS alone was almost exclusively in its hypophosphorylated form. Cells treated with either SB203580 or VEGF contained similar levels of hyperphosphorylated Rb, which was detected by its slower electrophoretic mobility. Treatment of the cells with both VEGF and SB203580 had a slightly stronger but not additive effect on Rb hyperphosphorylation. Rb completely shifted to the hyperphosphorylated form in cells incubated in complete growth medium. These results suggested that p38 MAP kinase had a negative effect on basal and

VEGF-stimulated Rb phosphorylation, and they correlated with the effect of this kinase on VEGF-induced endothelial cell proliferation.

Since the immediate regulators of Rb phosphorylation are cyclin/cdk complexes and cdk inhibitors (Sherr, 1994; Lin et al., 1996), we examined the expression of cyclin D1 and cdk inhibitors p21^{Waf1} and p27^{Kip1} under the conditions used to study Rb phosphorylation. Treatment of cells with SB203580 or VEGF caused a marked decrease in the level of p27^{Kip1} (Fig. 8). SB203580 and VEGF in combination did not have an additive effect on the expression of p27^{Kip1}. The levels of cyclin D1 and p21^{Waf1} did not change between treated and untreated cells. Although the expression and function of the three D-type cyclins (cyclin D1, D2, and D3) are cell type-specific (Sherr, 1994; Lavoie et al., 1995), we were unable to examine the effects of SB203580 or VEGF on the levels of cyclins D2 and D3 owing to the lack of specific antibodies for these cyclins. Taken together, our data indicated that the inhibitory effect of SB203580 on p38 MAP kinase activation, which is an early response to VEGF stimulation in HUVEC, was associated with enhanced Rb hyperphosphorylation. Phosphorylation of Rb results at least in part from a decrease in p27^{Kip1} levels. At present, we

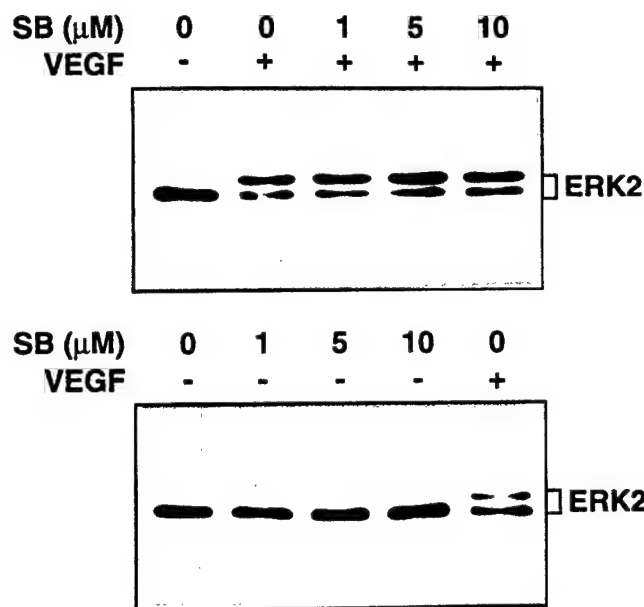


Fig. 7. Effects of SB203580 on VEGF-stimulated ERK activation in HUVEC. Serum-starved cells were incubated with either carrier (ethanol) or the indicated concentration of SB203580 (SB) for 20 min. The cells were left untreated or treated with VEGF (50 ng/ml) for 10 min. The electrophoretic mobility shift of ERK2 was detected by Western blotting. The results are representative of three independent experiments.

cannot rule out the possibility that SB203580 also targets other events regulating cell-cycle entry.

DISCUSSION

To study the mitogenic signaling events triggered by VEGF binding to its receptors, we have utilized HUVEC, which endogenously express both KDR and Flt1 VEGF receptors. These cells are a more appropriate model for studies of VEGF-induced cell signaling than nonendothelial cells overexpressing VEGF receptors. Here we show that VEGF activates four key signaling enzymes in HUVEC: ERK, p38 MAP kinase, PI 3-kinase, and p70 S6 kinase. Activation of these kinases is involved in VEGF-induced HUVEC proliferation.

VEGF simultaneously activates ERK and p38 MAP kinase but not JNK in HUVEC. Activation of ERK and p38 MAP kinases in response to VEGF was clearly demonstrated in this study by a characteristic electrophoretic mobility shift of the active kinases, an increase in phosphorylation of the kinases monitored with phosphospecific antibodies, and increased enzymatic activities toward exogenous substrates. VEGF-stimulated ERK and p38 MAP kinase enzymatic activities have been shown by others (D'Angelo et al., 1995; Seetharam et al., 1995; Rousseau et al., 1997). In this study, we were able to extend those observations by clearly associating VEGF-induced kinase phosphorylation with increased enzymatic activity. This is consistent with reports that phosphorylation of the kinases is required for their enzymatic activities (Payne et al., 1991; Han et al., 1994). We also show for the first time that VEGF, bFGF, and EGF share a common signaling

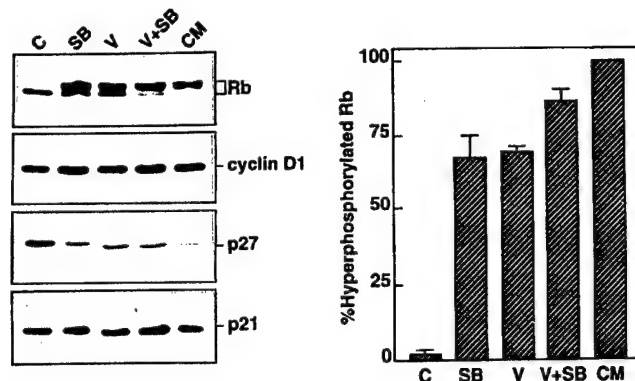


Fig. 8. Effects of SB203580 on cell-cycle regulatory proteins in HUVEC. **Left panel:** Serum-starved cells were cultured for 24 h in medium (DMEM/F-12 plus 1% FBS) (C), medium with 10 μ M SB203580 (SB), medium with 10 ng/ml VEGF (V), medium with VEGF (10 ng/ml) and 10 μ M SB203580 (V+SB), or with complete growth media (CM). Rb, cyclin D1, and cdk inhibitors p27^{Kip1} (p27) and p21^{Waf1} (p21) in cell lysates were detected by Western blot analysis with specific antibodies. p27^{Kip1} and p21^{Waf1} were analyzed on the same blot. The results represent one of three separated experiments. **Right panel:** Densitometric analysis of Rb in Western blots. The % hyperphosphorylated Rb is presented as the means \pm SE of three independent experiments. Abbreviations as in left panel.

pathway through ERK in HUVEC. The extent of ERK activation in VEGF-stimulated HUVEC was somewhat weaker than that in the cells treated with bFGF or EGF. This may be due to differences in receptor expression levels or intrinsic properties of the receptor kinases.

PI 3-kinase and p70 S6 kinase are activated by VEGF in HUVEC. VEGF stimulated a transient increase in PI 3-kinase activity. The activation of PI3-kinase involved tyrosine phosphorylation since kinase activity was measured using anti-phosphotyrosine immunoprecipitates. Phosphorylation of PI 3-kinase induced by VEGF has been previously shown in bovine aortic endothelial cells (Guo et al., 1995). Using the yeast two-hybrid method, Cunningham et al. (1995) have suggested that the p85 regulatory subunit of PI 3-kinase binds to Flt1. This evidence supports a conclusion that PI 3-kinase is involved in VEGF receptor signal transduction. In further support of this idea, we demonstrated that VEGF induced the activation of p70 S6 kinase, a downstream effector of PI 3-kinase (discussed below). By contrast, Abedi and Zachary (1997) did not detect increased PI 3-kinase activity upon VEGF stimulation in confluent human endothelial cell cultures. We believe the apparent discrepancy between this observation and our own is related to the density of cells at the time of treatment with VEGF; quiescent subconfluent HUVEC were used in this study. PI 3-kinase is differentially regulated by specific isoforms (α , β , or γ) of the p85 regulatory subunit (Baltenaperger et al., 1994; Harley et al., 1995; Crawley et al., 1996). At least two p85 isoforms (α and β) are expressed in HUVEC (data not shown). Whether the observed increase in PI 3-kinase activity is associated with p85 α or p85 β remains to be investigated.

Two approaches have been developed in order to dissect signal transduction pathways and to elucidate their physiological significance. One is to generate

dominant negative or constitutively active kinase mutants and to overexpress them in appropriate host cells. An alternative strategy is to use small cell-permeable inhibitors specific for particular protein kinases. Since the limited life span of HUVEC in culture precludes the possibility of establishing stable transfected cell lines, we chose to use specific kinase inhibitors to address the interactions between different pathways and to explore their roles in VEGF-induced human endothelial cell proliferation.

LY294002 inhibits PI 3-kinase activity *in vitro* and *in vivo*. Its specificity has been demonstrated by its lack of inhibitory effect on a number of ser/thr and tyrosine kinases, including PI 4-kinase, diacylglycerol kinase, src kinase, protein kinase C, MAP kinase, S6 kinases, and receptor tyrosine kinases for PDGF or EGF (Vlahos et al., 1994). In the present study, we found that inhibition of PI 3-kinase with LY294002 resulted in a blockade of p70 S6 kinase activation in response to VEGF. Similar results were observed when cells were treated with wortmannin, which is structurally distinct from LY294002 and is an inhibitor of PI 3-kinase at low concentrations (Ui et al., 1995). Our data suggest PI 3-kinase is an upstream component in the signaling cascade leading to the activation of p70 S6 kinase. This result is shown here for VEGF-stimulated endothelial cells, but it is consistent with previous studies of other growth factors, such as PDGF and insulin, in nonendothelial cell types (Chung et al., 1994; Petritsch et al., 1995). Recent reports have described a signal transduction complex which links PI 3-kinase, protein kinase B, and p70 S6 kinase with a newly identified 3-phosphoinositide-dependent protein kinase (PDK) (Pullen et al., 1998; Downward, 1998). Molecular dissection of this complex should provide further insights into the mechanisms of PI 3-kinase signaling.

The profound inhibitory effects of LY294002 and rapamycin on VEGF-induced HUVEC proliferation indicate that the PI 3-kinase/p70 S6 kinase pathway is essential for VEGF-stimulated cell mitogenesis. These results have established an association between activation of PI 3-kinase/p70 S6 kinase and their physiological roles in VEGF-stimulated human endothelial cells. Although PI 3-kinase and p70 S6 kinase have been implicated in mitogenesis (Fantl et al., 1992; Kozma and Thomas, 1994), their roles in mediating the mitogenic response of endothelial cells to VEGF have not been previously demonstrated. Since blocking activation of p70 S6 kinase with rapamycin inhibits the G1/S cell-cycle transition in some cell types (Dumont and Su, 1996; Luo et al., 1996), the inability of HUVEC to proliferate in response to VEGF following treatment with LY294002 is likely due in part to a loss in p70 S6 kinase function.

p38 MAP kinase has been shown to regulate cytokine biosynthesis (Lee et al., 1994) and to mediate cell migration (Guay et al., 1997; Rousseau et al., 1997). However, little is known about its role in cell proliferation. In this study, we show that p38 MAP kinase suppresses HUVEC proliferation. Inhibition of p38 MAP kinase with the specific inhibitor SB203580 enhanced HUVEC proliferation in the absence or the presence of VEGF. This effect was not due to interference with ERK activation, and it suggests that p38 MAP kinase

acts independently of the related MAP kinase. Studies of SB203580's effect on the cell-cycle progression clearly demonstrated that p38 MAP kinase regulated phosphorylation of Rb. The retinoblastoma tumor suppressor protein Rb regulates cell proliferation by controlling progression through the restriction point within the G1 phase of the cell cycle in mammalian cells (Sherr, 1996). Hypophosphorylated Rb functions as a transcription repressor that binds to transcription factors such as members of the E2F family. Phosphorylation of Rb releases E2F, which is important for the S phase entry (Lin et al., 1996). The present study therefore links p38 MAP kinase signaling to the cycle machinery in endothelial cells, and it indicates that the inhibition of p38 MAP kinase by SB203580 is associated with phosphorylation of Rb protein.

Rb phosphorylation is regulated by cyclin D- and E-associated cyclin-dependent kinases (cdks) in middle and late G1 phase (Sherr, 1995). Cyclin/cdk activities are negatively regulated by cdk inhibitors, such as p21^{Waf1} and p27^{Kip1} (Hunter, 1993). The protein level of p27^{Kip1} but not p21^{Waf1} decreased in SB203580-treated HUVEC. Thus, p38 MAP kinase appears to suppress the decline of p27^{Kip1}, which inhibits Rb phosphorylation and cell-cycle progression. However, the mechanism by which this suppression occurs is not clear. We cannot completely exclude the possibility that SB203580 affects targets in addition to p38 MAP kinase that regulate S phase entry. In addition, we show that ERK and p38 MAP kinase, two members of the MAP kinase family, play distinct roles in mediating the mitogenic response of HUVEC to VEGF. In contrast to p38 MAP kinase, ERK positively regulates HUVEC proliferation. A recent report by Kroll and Waltenberger et al. (1997) also showed that PD098059 inhibited VEGF-stimulated DNA synthesis in KDR-transfected porcine aortic endothelial cells.

In summary, this study shows that the activities of ERK, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation. Neither ERK nor PI 3-kinase/p70 S6 kinase activation alone is sufficient to stimulate the continuous proliferation of HUVEC. ERK and p38 MAP kinase play distinct and opposing roles in mediating the mitogenic response of HUVEC to VEGF. These results indicate that VEGF activates multiple signaling pathways to regulate endothelial cell proliferation. Our findings show that VEGF-receptor signaling exhibits a great degree of commonality with other receptor tyrosine kinases, and they suggest that targeting VEGF receptors on the endothelial cell surface may be a more appropriate therapeutic strategy than targeting intracellular signaling components that control VEGF-induced endothelial proliferation.

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VEGF SIGNALING PATHWAYS IN ENDOTHELIAL CELL MITOGENESIS

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Vascular endothelial cell growth factor (VEGF)/vascular permeability factor (VPF) is a specific mitogen for endothelial cells. Since 1991 it has been known that VEGF, like many other polypeptide growth factors, activates a receptor-associated tyrosine kinase in target cells. We have used normal human umbilical vein endothelial cells (HUVECs) rather than immortal endothelial or non-endothelial cell lines transfected with VEGF receptor cDNAs to study intracellular signaling pathways activated by VEGF in endothelial cell mitogenesis. We assessed the activation of enzyme signaling intermediates by phosphorylation state and by increased kinase activity towards substrates. In addition, we used specific inhibitors of signaling enzymes to evaluate their significance in VEGF-stimulated cell proliferation. Like all polypeptide growth factors that have been tested VEGF rapidly and transiently activated the mitogen-activated protein kinases (MAPKs) ERKs 1 and 2. It also activated p38 MAP kinase in subconfluent HUVECs, but it had no detectable effect on stress-activated protein kinase (SAPK)/jun N-terminal kinase (JNK). Phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream mediator p70 S6 kinase, phospholipase C gamma, and the delta and epsilon isoforms of protein kinase C were also activated by VEGF in HUVECs. By contrast, activation of the janus kinases (JAKs) and PKCs alpha and beta was not detected. The activities of ERKs 1 and 2, PI 3-kinase and p70 S6 kinase were specifically inhibited by PD98059, LY294002 and rapamycin, respectively, in VEGF-stimulated HUVECs, and each of the three inhibitors completely inhibited cell proliferation. There was no apparent cross-talk between PI 3-kinase or p70 S6 kinase and ERKs 1 and 2 or between the ERKs and p38 MAP kinase. Inhibition of p38 MAP kinase by the compound SB203580 resulted in the hyperphosphorylation of retinoblastoma (Rb) protein, and it stimulated HUVEC proliferation both in the presence and absence of VEGF. Our results indicate that the activities of the signaling enzymes ERKs 1 and 2, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation while p38 MAP kinase acts to suppress endothelial cell proliferation by inhibiting cell cycle progression. VEGF activates a multiplicity of signal transduction pathways in endothelial cells, but as none of these pathways is unique to VEGF, targeting VEGF receptors rather than intracellular signaling intermediates may be a more appropriate therapeutic strategy for controlling VEGF-induced cell proliferation *in vivo*.

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